

# Separation and Quantification of Two Fluoroquinolones in Serum by On-Line High-Performance Immunoaffinity Chromatography

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**To demonstrate that two structurally similar chemicals can be extracted from a complex matrix and then separated from each other on the basis of their relative affinities for an antibody, an automated column-switching system was used, incorporating on-line, high-performance immunoaffinity chromatography (HPIAC). A high-affinity monoclonal antibody (Mab Sara-95) against the fluoroquinolone sarafloxacin was covalently cross-linked to a protein G column and used to capture fluoroquinolones in fortified serum samples. Interference from matrix components adhering nonspecifically to the column was minimized by the insertion of a protein G cleanup column between the injection port and the Mab Sara-95 derivatized HPIAC column. Upon injection, serum samples containing the fluoroquinolones passed through both columns. The cleanup column detained serum components, that otherwise would bind nonspecifically to the HPIAC column, but allowed the fluoroquinolones to pass through unhindered to the HPIAC column. The fluoroquinolones were then eluted from the HPIAC column according to their relative affinities for the antibody, and individual peaks were monitored using fluorescence detection. By using an on-line cleanup column in tandem with an HPIAC column, the fluoroquinolones could be separated from the serum matrix and then separated from each other on the basis of their affinity for Mab Sara-95 without the use of organic solvents or reversed-phase liquid chromatography (RPLC). This method demonstrates true immunoaffinity separation of structurally related compounds in a complex matrix.**

Immunoassays have been successfully developed as alternatives to conventional chromatographic methods for the detection of pesticides, drug residues, and undesirable natural products that may contaminate food<sup>1</sup> or environmental<sup>2–5</sup> samples. The strength of these assays lies in their ability to detect compounds in complex

matrixes without the need for laborious sample cleanup procedures. This advantage allows them to be effective as rapid screens for identification of a small number of positive samples that may be present in the thousands of samples normally tested. However, immunoassays, in general, have not been used widely as analytical methods because most antibodies, even those of monoclonal origin, lack absolute immunospecificity for a single analyte.<sup>6</sup> Because an analyte is often a member of a “class” of compounds that exhibit similar structural/conformational shapes and electronic properties and because antibodies rely on these properties to detect compounds, broad cross-reactivity within a class of compounds is frequently observed. Although broad selectivity can be a problem for some immunoassay applications, the relatively nonselective nature of antibody binding can be advantageous for antibody-based sample-purification procedures in which all compounds within a given class must be extracted from the surrounding sample matrix.

To exploit the broad selectivity exhibited by most antibodies and to overcome the analytical difficulties associated with immunoassays, detection methods have been reported that use antibodies in on-line methods incorporating high-performance immunoaffinity chromatography (HPIAC).<sup>7–11</sup> In on-line HPIAC/HPLC methods, the immobilized antibodies “capture” structurally similar compounds from the sample matrix. Once the matrix has flushed to waste, the captured compounds are eluted from the HPIAC column to an analytical LC column for final separation of the analytes prior to detection. This second column is required because the compounds elute from the HPIAC columns as a single peak rather than as individual peaks. Insertion of a third column, such as a restricted access media (RAM) column, between the

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HPIAC and analytical columns, is often necessary to separate the analytes from interfering substances and to decouple the solution conditions used for the HPIAC column from the mobile phase that is optimal for the analytical column. Because the addition of RAM and analytical LC columns to the method lengthens the time required for each analysis, it would be advantageous to develop a detection method in which the HPIAC column could capture the analytes out of the surrounding matrix and then separate the analytes from each other. Such a method would avoid the laborious sample preparation necessary for traditional LC analyses and would circumvent the analytical difficulties associated with immunoassays. In addition to decreasing the labor/cost required for each analysis, this method could decrease or abolish the use of organic solvents.

Weak affinity chromatography (WAC) HPLC methods have been used to separate closely related molecules without the use of RAM and analytical LC columns.<sup>12–14</sup> However, WAC has been limited by the insufficient separation efficiency achieved and by the increased time required for solute analysis using isocratic elution.

In a previous study,<sup>15</sup> we demonstrated for the first time that a single, high-affinity monoclonal antibody was capable of separating a mixture of closely related small molecules (fluoroquinolones) in a chemical library and that the mechanism of separation was based on the individual affinities of the compounds for the antibody. We were hopeful that this phenomenon could be exploited to develop rapid, immunoaffinity-based analytical methods for multiresidue analyses in a variety of matrixes. However, when immunoaffinity separation of fluoroquinolones was attempted using samples such as milk,<sup>16</sup> tissues,<sup>17</sup> and serum (unpublished observations), components of these complex matrixes bound nonspecifically to the immunoaffinity column and coeluted with the less tightly held fluoroquinolones. Because of this interference, in these methods, an analytical LC column had to be used in tandem with an HPIAC column to separate coeluting serum components from the desired analytes. Interference from the sample matrix often hampers analyses and must be removed by off-line<sup>18,19</sup> or on-line<sup>20,21</sup> cleanup methods. In order for multi-analyte separation on an HPIAC column to be feasible for real-world samples, a means of removing interfering matrix components would have to be employed. Because our goal is to develop rapid, cost-effective detection methods, ideally, the cleanup procedure would employ an on-line, reusable column that is as robust as the HPIAC column.

In this study, we report removal of interfering serum matrix components using an on-line, reusable cleanup column in tandem

with an HPIAC column and development of an automated system capable of quantifying two fluoroquinolones in serum. The method does not require organic solvents for sample analysis or analytical LC column chromatography for separation of the analytes.

For this method, a cleanup column was packed with the same protein G-linked sorbent as was used to make the HPIAC column, but anti-fluoroquinolone antibodies were not covalently attached. When samples were automatically injected into the system with both columns in-line, components of the sample matrix that would have bound nonspecifically to the HPIAC column were first captured on the cleanup column. The fluoroquinolones and nonadhering sample components passed through the cleanup column to the HPIAC column. The covalently bound Mab Sara-95 on the HPIAC column then retained the fluoroquinolones, allowing nonadherent matrix components to pass through and elute to waste. The HPIAC column was then placed off-line. The cleanup column was washed with elution buffer (to remove the unwanted retained material) and reequilibrated with binding buffer. The HPIAC column was then placed in line with the cleanup column and elution buffer was passed through both columns, allowing each fluoroquinolone to elute from the HPIAC column according to its relative affinity for the antibody.

The results presented here demonstrate that a method employing an on-line cleanup column in tandem with a single HPIAC column is capable of separating two closely related compounds in a complex matrix without the need for analytical LC for final analysis. This method abolishes the use of organic solvents in both sample preparation and analyte detection. To our knowledge, this is the first report of resolving and quantifying structurally similar small molecules in a complex matrix using high-affinity monoclonal antibodies bound to a single HPIAC column.

## EXPERIMENTAL SECTION

**Fluoroquinolones.** Sarafloxacin (Abbott Laboratories, North Chicago, IL) and enrofloxacin (Bayer, Kansas City, MO) were gifts from their respective manufacturers. The stock standards were prepared by dissolving each fluoroquinolone in methanol at 1 mg/mL. The intermediate standards were made by diluting the stock standards in phosphate-buffered saline (PBS; 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M NaCl, pH 7.2) to obtain concentrations of 1, 5, and 10 µg/mL. These intermediate standards were then used to make analytical standards in PBS or to fortify serum at concentrations between 20 and 200 ng/mL.

**HPLC System.** The automatic column-switching capabilities of an Integral Microanalytical Workstation from PE Biosystems (Framingham, MA) were exploited in order to achieve separation of the fluoroquinolones from the serum matrix. The system consists of an autosampler, two HPLC pumps, three 10-port switching valves, two reagent syringe pumps, a fluorescence detector, and a variable-wavelength UV detector. Pump 1 delivered PBS and the binding buffer (0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 6.0), whereas pump 2 delivered the elution buffer (2% acetic acid, pH 2.5). For fluorescence detection, the excitation and emission wavelengths were set at 280 and 444 nm, respectively.

**High-Performance Immunoaffinity Chromatography (HPI-AC) Column.** The monoclonal antibody against sarafloxacin (Mab Sara-95; IgG1, κ), that was used to make the HPIAC column, was

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developed and characterized previously in our laboratory.<sup>22</sup> It was purified on a protein G column, dialyzed against PBS, and brought to a final concentration of 2 mg/mL prior to linkage to the HPIAC column sorbent. The HPIAC column was made by packing a PEEK cartridge (2.1 mmD × 30 mmL) with POROS XL media (a polystyrene/divinylbenzene polymer containing protein G covalently bound to its surface; PE Biosystems, Framingham, MA). Mab Sara-95 (3 mg) was adsorbed to the protein G surface and subsequently covalently cross-linked in place according to the manufacturer's instructions. The column was stored in PBS/0.02% sodium azide to prevent microbial contamination.

**Matrix Cleanup Columns.** Antibodies against compounds or proteins unrelated to sarafloxacin were used to prepare the matrix cleanup columns. Anti-furosemide (Mab furo-73; IgG1,  $\kappa$ )<sup>23</sup> and anti-aflatoxin M1 (Mab A1; IgG1,  $\kappa$ )<sup>24</sup> antibodies were each covalently bound to identical columns using the same procedure as that used to prepare the HPIAC column. A commercially available antihuman granulocyte colony stimulating factor (hG-CSF) polyclonal antiserum (R&D Systems, Inc., Minneapolis, MN) was also attached to a protein G column, but only 1 mL of a 0.9 mg/mL solution was covalently linked. An additional cleanup column was made containing the POROS XL packing material alone without treatment with an antibody or with the cross-linking and quenching reagents. All cleanup columns were stored in PBS/0.2% sodium azide to prevent microbial contamination.

**Column-Switching Events.** Removal of contaminating serum matrix components, capture of the fluoroquinolones on the HPIAC column, and separation of enrofloxacin and sarafloxacin was accomplished using the column-switching capabilities of the Integral system software. First, both the cleanup and the HPIAC columns were automatically placed in-line and equilibrated with PBS at 3 mL/min. The fluoroquinolones were injected onto the HPIAC column at 0.5 mL/min using the autosampler and a 100- $\mu$ L sample loop. The columns were washed with 25 column volumes of binding buffer. Matrix components that would have bound nonspecifically to sites on the HPIAC column were retained on the cleanup column, whereas the fluoroquinolones passed through the cleanup column and bound specifically to Mab Sara-95 that was covalently bound to the HPIAC column. Matrix components that did not bind to either column were flushed to waste.

After capturing the fluoroquinolones, the HPIAC column was switched off-line and the contaminating matrix components on the cleanup column were flushed to waste by eluting with elution buffer and reequilibrating with binding buffer. The HPIAC column was then switched in-line along with the cleanup column. Elution of the fluoroquinolones from the HPIAC column and separation of enrofloxacin and sarafloxacin prior to fluorescence detection was accomplished by a 10-column-volume isocratic elution step with elution buffer at 0.5 mL/min. After elution, both columns were reequilibrated with 30 column volumes (~3 mL) of PBS at 4 mL/min.

**Fortified Serum Samples.** *Preparation of Samples for the Standard Curve.* According to the manufacturer's dosing instruc-

tions for enrofloxacin, the minimum inhibitory concentration (MIC) values for a number of bacterial pathogens range between 16 and 2000 ng/mL. The concentrations of the fluoroquinolone in serum can range between 1100 (30 min posttreatment) and 180 ng/mL (12 h posttreatment). Therefore, serum samples (1 mL) were fortified at levels between 20 and 200 ng/mL with identical concentrations of both enrofloxacin and sarafloxacin. Following a 10-fold dilution of the samples with PBS, the samples were filtered through a 0.2- $\mu$ m filter directly into autosampler vials and analyzed.

*Preparation of Samples: Blind Study.* Serum samples (1 mL) were fortified at levels between 20 and 200 ng/mL with both enrofloxacin and sarafloxacin. Unlike the standards for the standard curve, enrofloxacin and sarafloxacin were not necessarily present in equal concentrations in these samples. Following a 10-fold dilution of the samples with PBS, the samples were filtered through a 0.2- $\mu$ m filter directly into autosampler vials and analyzed.

## RESULTS AND DISCUSSION

The fluoroquinolones enrofloxacin and sarafloxacin were selected to demonstrate that true immunoaffinity capture/separation chromatography can be used effectively to quantify structurally similar compounds in complex matrixes. On-line immunoaffinity capture has been used in tandem with analytical LC to extract and quantify compounds of interest in chemical libraries and in partially purified extracts of complex matrixes.<sup>7-11</sup> The method described here involving extraction and quantification of fluoroquinolones using an HPIAC column offers a number of advantages.

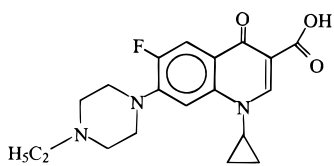
First, organic solvents are not required for sample cleanup. Simple dilution of the samples in PBS and filtration through a 0.2- $\mu$ m filter are the only steps necessary for sample preparation because of the selective nature of the anti-fluoroquinolone antibodies covalently linked to the HPIAC column. Second, a restricted access media (RAM) column is not required for separation of interfering matrix components from the analytes of interest. Placement of an on-line, reusable cleanup column prior to the immunoaffinity column effectively removes matrix components that otherwise would bind nonspecifically to the column packing material and interfere with sample analyses. Third, an analytical LC column is not necessary for analyte quantification because the structurally similar analytes are separated from each other by the HPIAC column on the basis of the differences in their relative affinities for the covalently attached antibody.

The structures of enrofloxacin and sarafloxacin are given in Figure 1. In a previous study involving development of Mab Sara-95,<sup>22</sup> the relative affinities of these fluoroquinolones were determined by enzyme-linked immunosorbent assay (ELISA) and reported as  $IC_{50}$  values. Sarafloxacin ( $IC_{50}$  = 8.2 ng/mL) exhibits an approximately 18-fold greater relative affinity for Mab Sara-95 than does enrofloxacin ( $IC_{50}$  = 154 ng/mL). In a recent study,<sup>15</sup> we demonstrated that this antibody, when immobilized on an HPIAC column, was capable of separating these two compounds on the basis of the difference between their relative affinities. To accomplish this, the traditional binding/elution buffer pair of PBS (pH 7)/12 mM HCl + 150 mM NaCl was abandoned and replaced with 0.1 M sodium phosphate (pH 6)/2% acetic acid. Further investigations revealed that other binding elution buffer pairs also accomplish separation of structurally related compounds. These

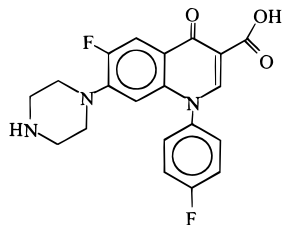
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Enrofloxacin



Sarafloxacin

Figure 1. Structures of the fluoroquinolones evaluated in this study.

pairs include PBS (pH 6)/2% acetic acid; 0.1 M ammonium acetate (pH 6)/2% acetic acid; and 0.1 M ammonium formate (pH 6)/2% formic acid (unpublished observations). Because an HPIAC column was capable of separating structural analogues in a chemical library, we expected that affinity-based separation and quantification of fluoroquinolones in complex matrixes would be feasible.

In subsequent studies, however, it became evident that quantification by immunoaffinity chromatography, alone, was not possible when these drugs were present in matrixes such as milk,<sup>16</sup> tissue,<sup>17</sup> and serum (unpublished observations). Figure 2A demonstrates that the chromatographic peak representing enrofloxacin is completely masked by a broad, coeluting peak containing serum components that bind to the HPIAC column. A number of additives were applied to each sample in an effort to prevent such binding. These additives included 0.1% Tween 20, 10% methanol, and a monoclonal antibody (Mab A1) against an unrelated compound, aflatoxin M1.<sup>24</sup> This antibody was used to determine if most of the nonspecific binding was due to interaction of matrix components with the antibody bound to the HPIAC column; however, addition of soluble Mab A1 did not prevent undesirable binding in this case. None of the additions to the samples significantly reduced nonspecific binding of matrix components to the immunoaffinity column (data not shown).

The POROS XL protein G-linked media that was used in these studies is just one of a number of commercially available immunoaffinity sorbents, all of which can have difficulties with undesirable binding of sample components. In addition to problems of nonspecific binding, many sorbents exhibit inefficient binding of antigens because the antibody is randomly immobilized onto the support material. Inefficient binding may be due to incorrect orientation of the antibody, deformation/inactivation of the antigen binding site as a result of multipoint attachment of the antibody, or steric hindrance of the antigen binding site by antibody molecules that are in close proximity.<sup>25</sup> Site-directed antibody immobilization strategies involving protein G,<sup>26</sup> protein A,<sup>27</sup> or metal-iminodiacetate<sup>28</sup> covalently bound to sorbents were developed to enhance antigen binding. Each of these resin

derivatives bind to sequences located in the C-terminal portion of the Fc region, thus orienting the antigen-combining site away from the resin to allow for maximal antigen binding. Although these strategies overcame some of the difficulties associated with randomly immobilized antibodies, problems involving undesirable binding remained. Both protein G and protein A bind to antibodies in biological samples, and metal-iminodiacetate binds to metal-binding proteins. These contaminants elute along with the desired analytes and interfere with analyses.

To overcome the difficulties associated with the protein G-linked sorbent, cleanup columns containing POROS XL media were developed and tested for their ability to reduce the amount of contaminating material eluting with the fluoroquinolones. In addition to interacting with the protein G-linked polymer, it was possible that biological macromolecules in the serum were interacting with other components of the HPIAC column, resulting in nonspecific binding. These components include (a) the cross-linking reagent that was used to attach Mab Sara-95 to protein G, (b) the quenching reagent that was used to deactivate the cross-linking reagent, and (c) the Sara-95 monoclonal antibody that was covalently bound to the sorbent. To determine what role these components may play in nonspecific binding, reusable cleanup columns were developed that were treated in a similar manner as the immunoaffinity column; however, rather than being linked to Mab Sara-95, the protein G-linked sorbent was covalently linked to antibodies against compounds unrelated to the fluoroquinolones. One additional cleanup column was made that contained the POROS XL media, alone, without derivatization with an antibody or treatment with the cross-linking and quenching reagents. The cleanup column containing the POROS XL media, alone, was the most effective at preventing unwanted binding, indicating that much of the nonspecific binding was the result of interactions of the serum matrix with the protein G-linked polymer. Figure 2B demonstrates that this cleanup column drastically decreased the amount of nonspecifically bound matrix material. Therefore, this cleanup column was used for subsequent studies.

Although the cleanup column removed most of the nonspecifically bound matrix components when one-half-dilute control serum was injected onto the column, sequential serum dilutions were performed to optimize the system. These studies demonstrated that the interfering peak obtained using one-tenth-dilute serum (Figure 2D) was substantially smaller than that obtained using one-half-dilute serum (Figure 2C), but was about equal to that obtained using one-twentieth-dilute serum (data not shown). Because further dilution did not improve the method, samples used in fortification studies were diluted one-tenth with PBS prior to analysis.

As can be observed by comparing parts D (one-tenth dilute serum) and E (PBS blank) of Figure 2, a low level of interfering matrix components eluted from the HPIAC column despite use of the cleanup column and dilution of the serum. Preliminary studies demonstrated that, if standard curves were generated using standards in PBS, the levels of enrofloxacin in fortified serum samples were consistently overestimated, particularly at the lowest fortification level (20 ng/mL). For samples fortified

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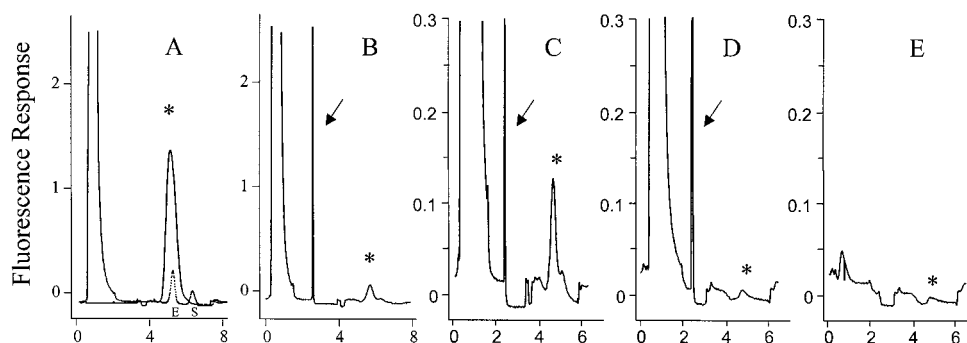


Figure 2. Removal of the interfering serum matrix peak. (A) Chromatogram (solid line) obtained by injecting 100  $\mu$ L of one-half-dilute serum without a cleanup column in line. The elution profile for enrofloxacin and sarafloxacin is also shown (dotted line). (B) Chromatogram obtained by injecting 100  $\mu$ L of one-half-dilute serum with the POROS XL cleanup column in line to trap serum components that would otherwise bind to the HPIAC column. For chromatograms in panels C, D, and E, the POROS XL cleanup column was used and 100  $\mu$ L of (C) one-half-dilute serum, (D) one-tenth-dilute serum, or (E) PBS was injected. In each panel, the \* marks the peak representing serum matrix components that interfere with fluoroquinolone analysis and the arrow marks the peak representing matrix components that bound to the cleanup column and were eluted to waste.

with both enrofloxacin and sarafloxacin at 20, 50, or 100 ng/mL, the corresponding recovery values for enrofloxacin were 133.5, 112.2, and 110.6%, whereas those for sarafloxacin were 109.6, 96.2, and 99.9%. To overcome this tendency to overestimate enrofloxacin levels, the standard curves for all subsequent studies were generated using standards in one-tenth-dilute serum.

Once the interfering peak was minimized, the elution parameters were investigated to optimize the separation of enrofloxacin and sarafloxacin while at the same time maintaining good peak shape for both compounds. The effect of the pH of the elution buffer on compound separation was investigated by altering the pH of the elution buffer with HCl or NaOH to obtain test solutions ranging between pH 2.0 and 2.8. When using the elution buffer at pH 2.0, sarafloxacin and enrofloxacin coeluted as one peak and no separation of the two compounds occurred. When using an elution buffer at pH 2.8, enrofloxacin eluted as a sharp peak, but sarafloxacin eluted as a broad mass that required more than 5 min for complete elution. When using an elution buffer at pH 2.5, distinct peaks for each fluoroquinolone were obtained. Therefore, an elution buffer (2% acetic acid) at pH 2.5 was used in subsequent studies.

The flow rate was varied between 0.3 and 1.5 mL/min using gradient or isocratic elution to optimize peak shape. Isocratic elution of the fluoroquinolones at 0.5 mL/min yielded the best chromatographic profiles for both compounds and was therefore used to generate the standard curve and to analyze fortified samples in the blind study. Representative chromatograms of a serum blank and of serum fortified with both enrofloxacin and sarafloxacin at 5 ng/mL are shown in parts A and B, respectively, of Figure 3.

The standard curve (in one-tenth-dilute serum) for enrofloxacin and sarafloxacin exhibited a lower limit of detection (LLD) of approximately 0.8 and 1.7 ng/mL, respectively (based on a signal-to-noise ratio of 5:1). Because the serum was diluted one-tenth prior to analysis, and because an injection volume of 100  $\mu$ L was used for each sample, these LLD values correspond to 80 and 170 pg per injected sample for enrofloxacin and sarafloxacin, respectively. Assay precision was good, with coefficients of variation less than 10%. Linear regression analysis gave the following equations: peak area = 700[ENRO] + 3046,  $r^2 = 0.999$  and peak area = 313[SARA] - 2042,  $r^2 = 0.999$ .

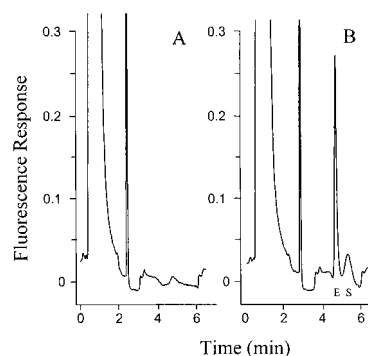


Figure 3. Representative chromatograms of (A) one-tenth-dilute serum blank or (B) one-tenth-dilute serum sample fortified at 5 ng/mL with both enrofloxacin and sarafloxacin. The POROS XL cleanup column was used in tandem with the HPIAC column.

In a blind study, enrofloxacin and sarafloxacin (at concentrations between 20 and 200 ng/mL) were assayed in 19 blind-coded fortified serum samples. Figure 4 demonstrates the correlation between fortification and recovery levels for (A) enrofloxacin and (B) sarafloxacin. The least-squares fit had a slope of 0.979 and a coefficient of determination ( $r^2$ ) of 0.995 for enrofloxacin analyses, whereas the least-squares fit had a slope of 0.990 and a  $r^2$  of 0.996 for sarafloxacin analyses. Individual recoveries for enrofloxacin ranged between 89 and 102% with a mean recovery of 94%, and individual recoveries for sarafloxacin ranged between 91 and 106% with a mean recovery of 98%.

The results presented here demonstrate that HPIAC is a powerful technique that is capable of separating and quantifying structurally similar compounds in real-world samples. Despite predictions that immunoaffinity chromatography would only be able to be used in extract cleanup procedures and would always have to be combined with other procedures (GC or reversed-phase HPLC) for final quantification,<sup>29</sup> this study involving high-affinity antibodies bound to an HPIAC column as well as previous studies involving weak affinity chromatography demonstrate that immunoaffinity chromatography alone can be used to extract and quantify small molecules in complex matrixes.

This method is particularly suited to analysis of pharmaceutical agents in clinical or veterinary samples. In these samples, the drug

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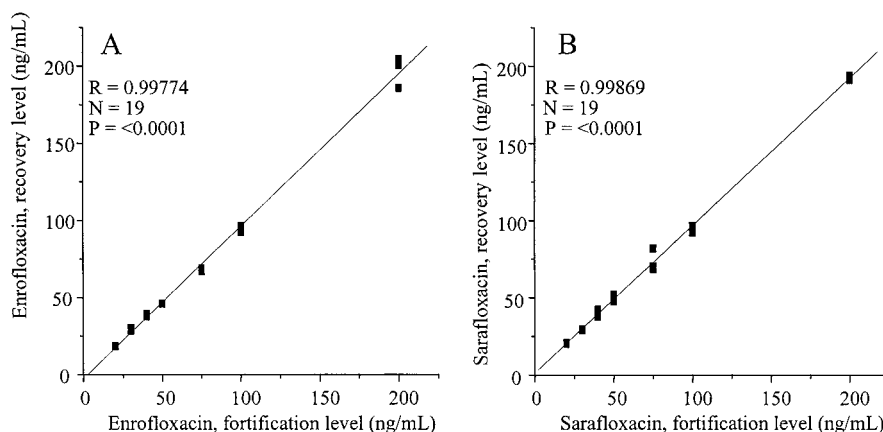


Figure 4. Comparison of fortification levels and recovery levels for (A) enrofloxacin and (B) sarafloxacin.

or drugs that are administered are known and sample analyses are performed in order to determine the level of the parent drug and/or its metabolites at any given point in time. By applying HPIAC to the sample, the parent drug can be separated from its metabolite(s) using an appropriate monoclonal antibody. If more than one class of drug has been administered, additional HPIAC columns can be placed in-line, each separating parent drugs from metabolites on the basis of the relative affinities of the compounds for the antibody on the column.

HPIAC analysis is also applicable to food and environmental samples. These types of samples potentially contain violative levels of any one of a number of legal or illegal compounds. Because the identity of the residues would not be known and because some of the residues may exhibit similar affinities for the antibody, each peak eluting from the HPIAC column may contain more than one compound. Coelution of similar compounds that bind to the same target molecule (i.e., an antibody or receptor) can hamper definitive identification of analytes. However, use of an on-line electrospray interface (ESI) mass spectrometer equipped with a time-of-flight (TOF) mass analyzer can circumvent this difficulty. Hsieh et al.<sup>30</sup> applied target-based selection coupled with ESI-TOF mass discrimination in an automated format to identify active compounds in compressed chemical libraries. In their study, desirable analytes were separated from undesirable interfering components using a size-exclusion column. The buffer was simply desalted prior to elution of the unresolved mixture of analytes to the mass spectrometer. Therefore, chromatographic separation of a mixture of coeluting compounds by reversed-phase chromatography was unnecessary prior to mass analysis by ESI-TOF.

Analogously, in our system, the fluoroquinolones are separated from undesirable interfering matrix components using a protein G-based cleanup column. The HPIAC column effectively captures the analytes using either a low-salt phosphate/acetic acid binding/elution buffer pair or binding/elution buffer pairs that are compatible with ESI-TOF analysis (i.e., 0.1 M ammonium acetate/2% acetic acid or 0.1 M ammonium formate/2% formic acid). On-line ESI-TOF analysis can then identify parent compounds and metabolites that coelute from the HPIAC on the basis of their mass differences. Thus, for samples potentially containing coeluting compounds, our method employing both a cleanup column

and an HPIAC column, when coupled with ESI-TOF mass analysis, would provide unambiguous compound identification without the need for chromatographic separation by reversed-phase chromatography. Such a method would not only allow for high-throughput screening (~5 min/sample) similar to that of immunoassay screening methods, but would also yield information about the identity of the captured residues, information comparable to that obtained by traditional HPLC methods. Future work will involve application of HPIAC/ESI-TOF to incurred residues in clinical samples.

## CONCLUSION

The production of high-affinity monoclonal antibodies made it possible to develop high-throughput, sensitive immunoassays capable of detecting low levels of residues in samples. Such antibodies were also useful as sample cleanup reagents when they were linked to immunoaffinity chromatography (IAC) columns and used to extract analytes from samples prior to final separation and quantification by HPLC methods. Automated HPIAC became feasible with the development of more rigid and higher efficiency column supports. This allowed IAC to be adapted to standard HPLC equipment, thus automating the purification step prior to final LC analysis. The method reported here demonstrates that HPIAC is capable of both purifying desired analytes from a complex mixture and separating structurally similar analytes prior to detection. Because of the ability of the HPIAC column to concentrate analytes, multiple injections of the same sample can be applied. Therefore, the method has a broad dynamic range because it is not limited with regard to sample size. Further studies investigating the development of recombinant antibodies exhibiting the desired affinities/specificities for HPIAC applications are ongoing in our laboratory.

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